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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/62, 15/31, 15/54, 1/21, A61K 38/45 — A2

(11) International Publication Number: WO 95/04151

(43) International Publication Date: 9 February 1995 (09.02.95)

(21) International Application Number: PCT/GB94/01647

(22) International Filing Date: 29 July 1994 (29.07.94)

(30) Priority Data:
PCT/GB93/01617 30 July 1993 (30.07.93) WO
(34) *Countries for which the regional or international application was filed:* GB et al.
9401787.8 31 January 1994 (31.01.94) GB

(71) Applicant (*for all designated States except US*): MEDEVA HOLDINGS B.V. [NL/NL]; Churchill-Laan 223, NL-1078 ED Amsterdam (NL).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): KHAN, Mohammed, Anjam [GB/GB]; Cambridge University Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB). HORMAECHE, Carlos, Estenio [GB/GB]; Cambridge University Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB). CHATFIELD, Steven, Neville [GB/GB]; Medeva Vaccine Research Unit, Dept. of Biochemistry, Imperial College of Science and Technology, London SW7 2AY (GB). DOUGAN, Gordon [GB/GB]; Medeva Vaccine Research Unit, Dept. of Biochemistry, Imperial College of Science and Technology, London SW7 2AY (GB).

(74) Agents: HUTCHINS, Michael, Richard et al.; Fry Heath & Spence, St. Georges House, 6 Yattendon Road, Horley, Surrey RH6 7BS (GB).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

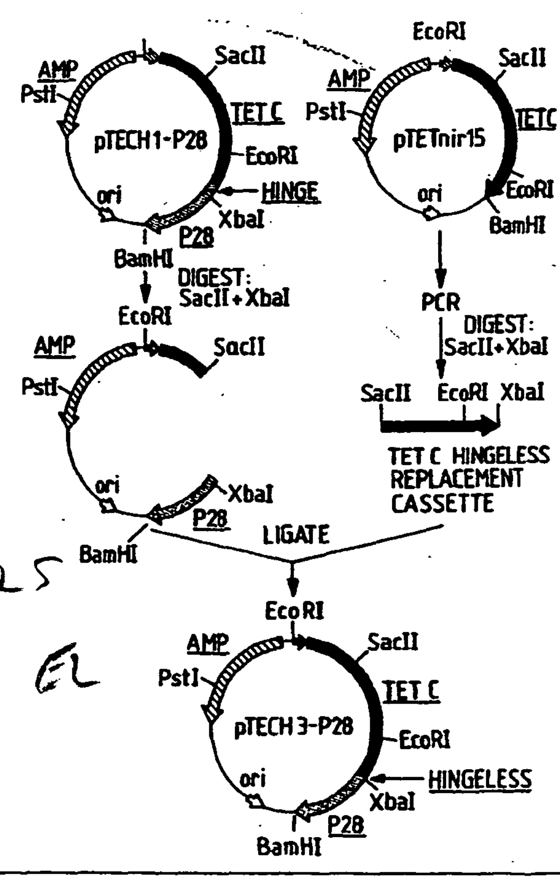
Published
Without international search report and to be republished upon receipt of that report.

(54) Title: VACCINE COMPOSITIONS

(57) Abstract

The invention provides a DNA construct comprising a DNA sequence encoding a fusion protein of the formula: TetC-(Z)$_a$-Het, wherein: TetC is the C fragment of tetanus toxin, or a protein comprising the epitopes thereof; Het is a heterologous protein, Z is an amino acid, and a is zero or a positive integer, provided that (Z)$_a$ does not include the sequence Gly-Pro. The invention also provides replicable expression vectors containing the constructs, bacteria transformed with the constructs, the fusion proteins *per se* and vaccine compositions formed from the fusion proteins or attenuated bacteria expressing the fusion proteins.

seems to
claim only fusion
protein as vaccines
— nothing to do with EL
or cell death



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VACCINE COMPOSITIONS

This invention relates to DNA constructs, replicable expression vectors containing the constructs, bacteria containing the constructs and vaccines containing the bacteria or fusion proteins expressed therefrom. More particularly, the invention relates to novel DNA constructs encoding the C-fragment of tetanus toxin, and to fusion proteins containing tetanus toxin C-fragment.

It is known to prepare DNA constructs encoding two or more heterologous proteins with a view to expressing the proteins in a suitable host as a single fusion protein. However, it has often been found that fusing two proteins together in this way leads to an incorrectly folded chimaeric protein which no longer retains the properties of the individual components. For example, the B-subunits of the Vibrio cholerae (CT-B) and E. coli (LT-B) enterotoxins are powerful mucosal immunogens but genetic fusions to these subunits can alter the structure and properties of the carriers and hence their immunogenicity (see M. Sandkvist et al. J. Bacteriol. 169, pp4570-6, 1987, Clements et al. 1990 and M. Lipscombe et al. Mol. Microbiol. 5, pp 1385, 1990). Moreover, many heterologous proteins expressed in bacteria ar not produced in soluble

properly folded or active forms and tend to accumulate as insoluble aggregates (see C. Schein et al. Bio/Technology 6, pp 291-4, 1988 and R. Halenbeck et al. Bio/Technology 7, pp 710-5, 1989.

In our earlier unpublished international patent application PCT/GB93/01617, it is disclosed that by providing a DNA sequence encoding tetanus toxin C-fragment (TetC) linked via a "hinge region" to a second sequenc encoding an antigen, the expression of the sequence in bacterial cells is enhanced relative to constructs wherein the C-fragment is absent. For example, the expression level of the full length P28 glutathione *S*-tranferase protein of S. mansoni when expressed as a fusion to TetC from the nirB promoter was greater than when the P28 protein was expressed alone from the nirB promoter. The TetC fusion to the full length P28 protein of S. mansoni was soluble and expressed in both E. coli and S. typhimurium. In addition, the TetC-P28 fusion protein was capable of being affinity purified by a glutathione agarose matrix, suggesting that the P28 had folded correctly to adopt a conformation still capable of binding to its natural substrate. It was previously considered that a hinge region, which typically is a sequence encoding a high proportion of proline and/or glycine amino acids, is essential for promoting the independent folding of both the TetC and the antigenic protein fused thereto. However, it has now been discovered, surprisingly in view f the previous studies on CT-B and LT-B referred to ab ve, that

when the hing region is omitted between the TetC and a second antigen such as P28, the proteins making up the fusion do exhibit correct folding as evidenced by affinity purification on a glutathione agarose matrix.

Accordingly, in a first aspect, the invention provides a DNA construct comprising a DNA sequence encoding a fusion protein of the formula TetC-$(Z)_a$-Het, wherein TetC is the C fragment of tetanus toxin, or a protein comprising the epitopes thereof; Het is a heterologous protein; Z is an amino acid, and a is zero or a positive integer, provided that $(Z)_a$ does not include the sequence Gly-Pro.

Typically $(Z)_a$ is a chain of 0 to 15 amino acids, for example 0 to 10, preferably less than 6 and more preferably less than 4 amino acids.

In one embodiment $(Z)_a$ is a chain of two or three amino acids, the DNA sequence for which defines a restriction endonuclease cleavage site.

In another embodiment, a is zero.

Usually the group $(Z)_a$ will not contain, simultaneously, both glycine and proline, and generally will not contain either glycine or proline at all.

In a further embodiment, $(Z)_a$ is a chain of amino acids provided that when a is 6 or more, $(Z)_a$ does not contain glycine or proline.

The group $(Z)_a$ may be a chain of amino acids substantially devoid of biological activity.

In a second aspect the invention provid s a replicable expression vector, for example suitabl for use in

bacteria, containing a DNA construct as hereinbefore defined.

In an ther aspect, the invention provides a host (e.g. a bacterium) containing a DNA construct as hereinbefore defined, the DNA construct being present in the host either in the form of a replicable expression vector such as a plasmid, or being present as part of the host chromosome, or both.

In a further aspect, the invention provides a fusion protein of the form TetC-$(Z)_a$-Het as hereinbefore defined, preferably in substantially pure form, said fusion protein being expressible by a replicable expression vector as hereinbefore defined.

In a further aspect the invention provides a process for the preparation of a bacterium (preferably an attenuated bacterium) which process comprises transforming a bacterium (e.g. an attenuated bacterium) with a DNA construct as hereinbefore defined.

The invention also provides a vaccine composition comprising an attenuated bacterium, or a fusion protein, as hereinbefore defined, and a pharmaceutically acceptable carrier.

The heterologous protein "Het" may for example be a heterologous antigenic sequence, e.g. an antigenic sequence derived from a virus, bacterium, fungus, yeast or parasite.

Examples of viral antigenic sequences are sequences derived from a type of human immunodeficiency virus (HIV) such as HIV-1 or HIV-2, the CD4 rec ptor binding sit from

HIV, for example from HIV-1 or -2., hepatitis A, B or C virus, human rhinovirus such as type 2 or type 14, Herpes simplex virus, poliovirus type 2 or 3, foot-and-mouth disease virus (FMDV), rabies virus, rotavirus, influenza virus, coxsackie virus, human papilloma virus (HPV), for example the type 16 papilloma virus, the E7 protein thereof, and fragments containing the E7 protein or its epitopes; and simian immunodeficiency virus (SIV).

Examples of antigens derived from bacteria are those derived from Bordetella pertussis (e.g. P69 protein and filamentous haemagglutinin (FHA) antigens), Vibrio cholerae, Bacillus anthracis, and E.coli antigens such as E.coli heat Labile toxin B subunit (LT-B), E.coli K88 antigens, and enterotoxigenic E.coli antigens. Other examples of antigens include the cell surface antigen CD4, Schistosoma mansoni P28 glutathione *S*-transferase antigens (P28 antigens) and antigens of flukes, mycoplasma, roundworms, tapeworms, Chlamydia trachomatis, and malaria parasites, eg. parasites of the genus plasmodium or babesia, for example Plasmodium falciparum, and peptides encoding immunogenic epitopes from the aforementioned antigens.

Particular antigens include the full length Schistosoma mansoni P28, and oligomers (e.g. 2, 4 and 8-mers) of the immunogenic P28 aa 115-131 peptide (which contains both a B and T cell epitope), and human papilloma virus E7 protein, Herp s simplex antigens, foot and mouth diseas virus antigens and simian immunodeficiency virus

antigens.

The DNA constructs of the present invention may contain a promoter whose activity is induced in response to a change in the surrounding environment. An example of such a promoter sequence is one which has activity which is induced by anaerobic conditions. A particular example of such a promoter sequence is the nirB promoter which has been described, ~~for example~~ in International Patent Application PCT/GB92/00387. The nirB promoter has been isolated from E.coli, where it directs expression of an operon which includes the nitrite reductase gene nirB (Jayaraman et al, J. Mol. Biol. 196, 781-788, 1987), and nirD, nirC, cysG (Peakman et al, Eur. J. Biochem. 191, 315323, 1990). It is regulated both by nitrite and by changes in the oxygen tension of the environment, becoming active when deprived of oxygen, (Cole, Biochem, Biophys. Acta. 162, 356-368, 1968). Response to anaerobiosis is mediated through the protein FNR, acting as a transcriptional activator, in a mechanism common to many anaerobic respiratory genes. By deletion and mutational analysis the part of the promoter which responds solely to anaerobiosis has been isolated and by comparison with other anaerobically regulated promoters a consensus FNR-binding site has been identified (Bell et al, Nucl, Acids. Res. 17, 3865-3874, 1989; Jayaraman et al, Nucl, Acids, Res. 17, 135-145, 1989). It has also been shown that the distance between the putative FNR-binding site and the -10 hom logy region is critical (Bell et al, M lec. Microbiol.4, 1753-

1763, 1990). It is therefore preferred to use only that part of the nirB promoter which responds solely to anaerobiosis. As used herein, references to the nirB promoter refer to the promoter itself or a part or derivative thereof which is capable of promoting expression of a coding sequence under anaerobic conditions. The preferred sequence, and which contains the nirB promoter is:

AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGG
TAGGCGGTAGGGCC (SEQ ID NO: 1)

In a most preferred aspect, the present invention provides a DNA molecule comprising the nirB promoter operably linked to a DNA sequence encoding a fusion protein as hereinbefore defined.

In another preferred aspect of the invention, there is provided a replicable expression vector, suitable for use in bacteria, containing the nirB promoter sequence operably linked to a DNA sequence encoding a fusion protein as hereinbefore defined.

The DNA molecule or construct may be integrated into the bacterial chromosome, e.g. by methods known per se, and thus in a further aspect, the invention provides a bacterium having in its chromosome, a DNA sequence or construct as hereinbefore defined.

Stable expression of the fusion protein can be obtained in vivo. The fusion protein can be expressed in an attenuated bacterium which can thus be used as a vaccine.

The attenuated bacterium may be selected from the gen ra Salmonella, Bordetella, Vibrio, Haemophilus, Neisseria and Yersinia. Alternatively, the attenuated bacterium may be an attenuated strain of enterotoxigenic Escherichia coli. In particular the following species can be mentioned: S.typhi - the cause of human typhoid; S.typhimurium - the cause of salmonellosis in several animal species; S.enteritidis - a cause of food poisoning in humans; S.choleraesuis - a cause of salmonellosis in pigs; Bordetella pertussis - the cause of whooping cough; Haemophilus influenzae - a cause of meningitis; Neisseria gonorrhoea the cause of gonorrhoea; and Yersinia - a cause of food poisoning.

Examples of attenuated bacteria are disclosed in, for example EP-A-0322237 and EP-A-0400958, the disclosures in which are incorporated by reference herein.

An attenuated bacterium containing a DNA construct according to the invention, either present in the bacterial chromosome, or in plasmid form, or both, can be used as a vaccine. Fusion proteins (preferably in substantially pure form) expressed by the bacteria can also be used in the preparation of vaccines. For example, a purified TetC-P28 fusion protein in which the TetC protein is linked via its C-terminus to the P28 protein with no intervening hinge region has been found to be immunogenic on its own. In a further aspect therefore, the invention provides a vaccine compositi n comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, an attenuated

bacterium or fusion protein as h reinbefore defined.

The vaccine may comprise one or mor suitable adjuvants.

The vaccine is advantageously presented in a lyophilised form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", Cellulose acetate, Cellulose acetate phthalate or Hydroxypropylmethyl Cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramammary administration.

The attenuated bacterium containing the DNA construct or fusion protein of the invention may be used in the prophylactic treatment of a host, particularly a human host but also possibly an animal host. An infection caused by a microorganism, especially a pathogen, may therefore be prevented by administering an effective dose of an attenuated bacterium acc rding to the invention. The bacterium then expresses the fusi n protein which is

capable of raising antibody to the micro-organism. The dosage empl yed will be dependent on vari us factors including the size and weight of the host, the type of vaccine formulated and the nature of the fusion protein.

An attenuated bacterium according to the present invention may be prepared by transforming an attenuated bacterium with a DNA construct as hereinbefore defined. Any suitable transformation technique may be employed, such as electroporation. In this way, an attenuated bacterium capable of expressing a protein or proteins heterologous to the bacterium may be obtained. A culture of the attenuated bacterium may be grown under aerobic conditions. A sufficient amount of the bacterium is thus prepared for formulation as a vaccine, with minimal expression of the fusion protein occurring.

The DNA construct may be a replicable expression vector comprising the nirB promoter operably linked to a DNA sequence encoding the fusion protein. The nirB promoter may be inserted in an expression vector, which already incorporates a gene encoding one of the heterologous proteins (e.g. the tetanus toxin C fragment), in place of the existing promoter controlling expression of the protein. The gene encoding the other heterologous protein (e.g. an antigenic sequence) may then be inserted. The expression vector should, of course, be compatible with the attenuated bacterium into which the vector is to be inserted.

The expressi n vector is provided with appropriate

transcriptional and translational control elements including, besides th nirB promoter, a transcriptional termination site and translational start and stop codons. An appropriate ribosome binding site is provided. The vector typically comprises an origin of replication and, if desired, a selectable marker gene such as an antibiotic resistance gene. The vector may be a plasmid.

The invention will now be illustrated but not limited, by reference to the following examples and the accompanying drawings, in which:

Figure 1 is a schematic illustration of the construction of plasmid pTECH1;

Figure 2 illustrates schematically the preparation of the plasmid pTECH1-28 from the starting materials pTECH1 and PUC19-P28;

Figure 3 illustrates schematically the preparation of the plasmid pTECH3-P28 from the starting materials plasmids pTECH1-P28 and pTETnir15;

Figures 4 and 5 are western blots obtained from bacterial cells harbouring the pTECH3-P28 construct; and

Figure 6 illustrates the glutathione affinity purification of TetC fusions as determined by SDS-PAGE and Coomassie Blue Staining.

In accordance with the invention a vector was constructed to allow genetic fusions to the C-terminus of the highly immunogenic C fragment of tetanus toxin, without the use f a heterologous hinge domain. A fusion was constructed, with the gen encoding the protective 28kDa

glutathione *S*-transferase from Schistos ma mansoni. The recombinant vector was transformed into Salmonella typhimurium (SL338; rm^{+}). The resulting chimeric protein was stably expressed in a soluble form in salmonella as assessed by western blotting with fragment C and glutathione *S*-transferase antisera. Furthermore it was found that the P28 component of the fusion retains th capacity to bind glutathione.

The construction of the vector and the properties of the fusion protein expressed therefrom are described in more detail below.

EXAMPLE 1

Preparation of pTECH1

The preparation of pTECH1, a plasmid incorporating the nirB promoter and TetC gene, and a DNA sequence encoding a hinge region and containing restriction endonuclease sites to allow insertion of a gene coding for a second or guest protein, is illustrated in Figure 1. Expression plasmid pTETnir15, the starting material shown in Figure 1, was constructed from pTETtac115 (Makoff *et al*, Nucl. Acids Res. 17 10191-10202, 1989); by replacing the EcoRI-ApaI region (1354bp) containing the lacI gene and tac promoter with the following pair of oligos 1 and 2:

Oligo-1 5'AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAAT
CGTTAAGGTAGGCGGTAGGGCC-3' (SEQ ID NO: 2)

Oligo-2 3'-GTCCATTTAAACTACATGTAGTTTACCATGGGGAACGACTTA
GCAATTCCATCCGCCATC-5' (SEQ ID NO: 3)

Th oligonucleotides w re synthesised on a Pharmacia Gene Assembler and the resulting plasmids confirmed by sequencing (Makoff *et al*, Bio/Technology 7, 1043-1046, 1989).

The pTETnir15 plasmid was then used for construction of the pTECH1 plasmid incorporating a polylinker region suitable as a site for insertion of heterologous DNA to direct the expression of fragment C fusion proteins. pTETnir15 is a known pAT153-based plasmid which directs the expression of fragment C. However, there are no naturally occurring convenient restriction sites present at the 3'-end of the TetC gene. Therefore, target sites, preceded by a hinge region, were introduced at the 3'-end of the TetC coding region by means of primers SEQ ID NO: 4 and SEQ ID NO: 5 tailored with "add-on" adapter sequences (Table 1), using the polymerase chain reaction (PCR) [K. Mullis *et al*, Cold Spring Harbor Sym. Quant. Biol. 51, 263-273 1986]. Accordingly, pTETnir15 was used as a template in a PCR reaction using primers corresponding to regions covering the SacII and BamHI sites. The anti-sense primer in this amplification was tailored with a 38 base 5'-adaptor sequence. The anti-sense primer was designed so that a sequence encoding novel XbaI, SpeI and BamHI sites were incorporated into the PCR product. In addition, DNA sequences encoding additional extra amino acids including proline were incorporated (the hinge regions) and a translation st p codon signal in frame with the fragment C open reading frame.

The PCR product was gel-purified and digested with <u>SacII</u> and <u>BamHI</u>, and cloned into the residual 2.8 kb vector pTETnir15 which had previously been digested by <u>SacII</u> and <u>BamHI</u>. The resulting plasmid purified from transformed colonies and named pTECH 1 is shown in Figure 1. Heterologous sequences such as the sequence encoding the <u>Schistosoma</u> <u>mansoni</u> P28 glutathione *S*-transferase (P28) were cloned into the <u>XbaI</u> <u>SpeI</u> and <u>BamHI</u> sites in accordance with known methods.

The DNA sequence of the plasmid pTECH1 is shown in the sequence listing as SEQ ID NO: 6.

<u>TABLE 1</u>

<u>DNA SEQUENCES OF OLIGONUCLEOTIDES UTILISED IN THE CONSTRUCTION OF THE TETC-HINGE VECTORS</u>

<u>A). Primer 1.</u> Sense PCR (21mer). (SEQ ID NO: 4)

SacII

5'AAA GAC TCC GCG GGC GAA GTT -3'

TETANUS TOXIN C FRAGMENT SEQ.

<u>B).Primer 2.</u> Anti-Sense PCR Primer (64mer). (SEQ ID NO: 5)

BamHI STOP SpeI XbaI [illegible]

5'- CTAT GGA TCC <u>TTA</u> ACT AGT GAT TCT AGA [illegible]

GTC GTT GGT CCA ACC TTC ATC GGT -3'

TETANUS TOXIN C FRAGMENT SEQ. 3'-END

<u>EXAMPLE 2</u>

<u>Construction of pTECH1-P28</u>

A P28 gene expression cassette was produced by PCR

using pUC19-P28 DNA (a kind gift from Dr R Pierce, Pasteur Institute, Lille) as template. Oligonucleotide primers were designed to amplify the full length P28 gene beginning with the start codon and terminating with the stop codon. In addition, the sense and antisense primers were tailored with the restriction sites for XbaI and BamHI respectively. The primers are shown in the sequence listing as SEQ ID NO: 7 and SEQ ID NO: 8.

The product was gel-purified and digested with XbaI and BamHI and then cloned into pTECH1 which had previously been digested with these enzymes and subsequently gel-purified. The DNA sequence of pTECH1 - P28 is shown in sequence listing as SEQ ID NO: 9.

Expression of the TetC-Hinge-P28 fusion protein

Several bacterial strains, namely *S. typhimirium* strains SL 5338 (A. Brown *et al*, J.Infect.Dis. 155, 86-92, 1987) and SL3261 and *E. coli* (TG2) were transformed with pTECH1-P28 by means of electroporation. SL3261 strains harbouring the pTECH1-P28 plasmid have been deposited at the National Collection of Type Cultures, 61 Colindale Avenue, London, NW9 5HT, UK under the accession number NCTC 12833. A strain of SL3261 containing the pTECH1 plasmid has been deposited under accession number NCTC 12831. The identity of recombinants was verified by restriction mapping of the plasmid DNA harboured by the cells. Further expression of the TetC-P28 fusion protein was then evaluated by SDS-PAGE and western blotting f bacterial cells harbouring the construct It was found that the

fusion protein remains soluble, cross-reacts with antisera to both TetC and P28, and is also of the expect d molecular weight, 80kDal, for a full length fusion.

The fusion protein was stably expressed in E.coli (TG2) and S. typhimurium (SL5338,SL3261) as judged by SDS-PAGE and western blotting. Of interest was a band of 50kDal which co-migrates with the TetC-Hinge protein alone and cross-reacts exclusively with the anti-TetC sera is visible in a western blot. As the codon selection in the hinge region has been designed to be suboptimal, the rare codons may cause pauses during translation which may occasionally lead to the premature termination of translation, thus accounting for this band.

Affinity purification of the TetC-P28 fusion

Glutathione is the natural substrate for P28, a glutathione *S*-transferase. The amino acid residues involved in binding glutathione are thought to be spatially separated in the primary structure of the polypeptide and brought together to form a glutathione binding pocket in the tertiary structure (P. Reinemer *et al.* EMBO, J8, 1997-2005, 1991). In order to gauge whether the P28 component of the fusion has folded correctly to adopt a conformati n capable of binding glutathione, its ability to be affinity purified on a glutathione-agarose matrix was tested. The results obtained (not shown) demonstrated that TetC-P28 can indeed bind to the matrix and the binding is reversible, as the fusion can be competitively eluted with free glutathione.

EXAMPLE 3

Construction of pTECH3-P28

The plasmid pTECH1-P28 directs the expression of the S. mansoni P28 protein as a C-terminal fusion to fragment C from tetanus toxin separated by a heterologous hinge domain. Expression of the fusion protein is under the control of the nirB promoter. The vector pTECH3-P28 was in part constructed from the plasmid pTETnir15 by the polymerase chain reaction (PCR) using the high fidelity thermostable DNA polymerase from Pyrococcus fusorius, which possesses an associated 3'5' exonuclease proofreading activity. The sequence of steps is summarised in Figure 5. In order to generate a TetC-hingeless replacement cassette, the segment of DNA from the unique SacII site within the TetC gene to the final codon was amplified by means of the PCR reaction, using pTETnir15 as template DNA. The primers used in the PCR amplification are shown in the sequence listing as SEQ ID NO: 10 and SEQ ID NO: 11. The antisense primer in this amplification reaction was tailored with an XbaI recognition sequence.

The amplification reaction was performed according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The product was gel-purified, digested with SacII and XbaI, and then cloned into the residual pTECH1-P28 vector which had been previously digested with the respective enzymes SacII and XbaI. The resulting vector was designated pTECH3-P28. The DNA sequence f pTECH3-P28 is shown in the sequenc listing as SEQ ID NO: 12.

EXAMPLE 4

Transformation of S. typhimurium SL5338 (galE r^-m^+) with pTECH3-P28, and Analysis of the Transformants

S. typhimurium SL5338 (galE r^-m^+) were cultured in either L or YT broth and on L-agar with ampicillin (50 g/ml) if appropriate and were transformed with the pTECH3-P28 plasmid. The transformation protocol was based on the method described by MacLachlan and Sanderson. (MacLachlan PR and Sanderson KE, 1985. Transformation of Salmonella typhimurium with plasmid DNA : differences between rough and smooth strains. J. Bacteriology 161, 442-445).

A 1ml overnight culture of S. typhimurium SL5338 (r^-m^+; Brown A, Hormaeche CE, Demarco de Hormaeche R, Dougan G, Winther M, Maskell D, and Stocker BAD, 1987. J. Infect.Dis. 155, 86-92) was used to inoculate 100 ml of LB broth and shaken at 37°C until the culture reached OD_{650} = 0.2. The cells were harvested at 3000 x g and resuspended in 0.5 volumes if ice-cold 0.1M $MgCl_2$. The cells were pelleted again and resuspended in 0.5 volumes of ice-cold $CaCl_2$. This step was repeated once more and the cells resuspended in 1 ml of 0.1M $CaCl_2$ to which was added 50 µl of TES (50 mM Tris, 10 mM EDTA, 50 mM NaCl, pH 8.0). The cells were incubated on ice for 45 to 90 minutes. To 150µl of cells was added 100ng of plasmid DNA in 1 - 2µl. The mixture was incubated on ice for 30 minutes prior to heat-shock at 42°C for 2 minutes, and immediate reincubation on ice for 1 minute. To the transformed mixture was added 2 ml f LB broth and incubated for 1.5 hours to allow

expression of th ampicillin drug resistance gen , B-lactamase. F llowing incubation 20 µl and 200 µl of c lls were spread on to LB agar plates containing 50 µg/ml of ampicillin. The plates were dried and incubated at 37°C overnight.

The identity of recombinants was verified by restriction mapping of the plasmid DNA and by western blotting with antisera directed against TetC and P28.

SDS-PAGE and Western Blotting

Expression of the TetC fusions was tested by SDS-PAGE and western blotting. S. typhimurium SL5338 (galE r^-m^+) bacterial cells containing the pTECH3-P28 plasmid and growing in mid-log phase, with antibiotic selection, were harvested by centrifugation and the proteins fractionated by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane by electroblotting and reacted with either a polyclonal rabbit antiserum directed against TetC or the full length P28 protein. The blots were then probed with goat anti-rabbit Ig conjugated to horse-radish peroxidase (Dako, High Wycombe, Bucks, UK) and developed with 4-chloro-1-napthol). The results of the western blotting experiments are shown in Figures 4 and 5; Figure 4 illustrating the results of probing with rabbit anti-TetC polyclonal antiserum and Figure 5 illustrates the results of probing with rabbit anti-P28 polyclonal antiserum. In each case lanes 1, 2 and 3 are independent clones of SL5338 (pTECH3-P28), lanes 4, 5 and 6 are SL5338 (pTECH1-P28) and

lane 7 is SL5338 (pTETnir15). The mol cular weight markers are indicated. From the results, it is evident that the fusion protein remains soluble, reacts with antisera to both TetC and P28, and is also of the expected molecular weight, 80 kDal, for a full length fusion (Figure 4). Furthermore the fusion protein appears to be stably expressed.

Glutathione-Agarose Affinity Purification

Glutathione is the natural substrate for P28, a glutathione *S*-transferase. The amino acid residues involved in binding glutathione are thought to be spatially separated in the primary structure of the polypeptide and brought together to form a glutathione binding pocket in the tertiary structure. In order to gauge whether the P28 component of the fusion has folded correctly to adopt a conformation capable of binding glutathione, we tested its ability to be affinity purified on a glutathione agarose matrix.

Bacterial cells containing pTECH3-P28 and expressing the TetC full length P28 gene fusion were grown to log phase, chilled on ice, and harvested by centrifugation at 2500 x g for 15 min at 4C. The cells were resuspended in 1/15th the original volume of ice-cold phosphate buffered saline (PBS) and lysed by sonication in a MSE Soniprep 150 (Gallenkamp, Leicester, UK). The insoluble material was removed by centrifugation and to the supernatant was added 1/6 v lume of a 50% slurry of pre-swollen glutathi ne-agarose b ads (Sigma, Poole, Dorset, UK). After mixing

gently at ro m temperature for 1 hour the beads were collected by centrifugati n at 1000 x g for 10 secs. The supernatant was discarded and the beads resuspended in 20 volumes of cold PBS-0.5% Triton X100 and the beads collected again by centrifugation. The washing step was repeated three more times. The fusion protein was eluted by adding 1 volume of SDS-PAGE sample buffer. For comparison purposes, a similar procedure was followed with bacterial cells containing the PTECH1-P28 plasmid from which TetC-hinge-P28 fusion protein is expressed. Extracts from clones containing either plasmid were compared using SDS-PAGE and the results are shown in Figure 6. In Figure 6, lanes 1, 2 and 3 are clones of SL5338 (pTECH1-P28) whereas lanes 4, 5 and 6 are independent clones of SL 5338 (pTECH3-P28).

The results suggest that the TetC-P28 fusion protein can indeed bind to the matrix and the binding is reversible regardless of the absence of a heterologous hinge domain (data not shown) It is possible that a peptide sequence present at the C-terminus of TetC may in fact impart flexibility to this particular region.

INSDOCID: <WO__9504151A2_I_>

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: MEDEVA HOLDINGS BV
(B) STREET: CHURCHILL-LAAN 223
(C) CITY: AMSTERDAM
(E) COUNTRY: THE NETHERLANDS
(F) POSTAL CODE (ZIP): 1078 ED

(ii) TITLE OF INVENTION: VACCINES

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/GB93/01617
(B) FILING DATE: 30-JUL-1993

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9401787.8
(B) FILING DATE: 31-JAN-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURE:
(A) NAME/KEY: promoter
(B) LOCATION: 1..61

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AATTCAGGTA AATTTGATGT ACATCAAATG GTACCCCTTG CTGAATCGTT AAGGTAGGCG 60

GTAGGGCC 68

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTCAGGTA AATTTGATGT ACATCAAATG GTACCCCTTG CTGAATCGTT AAGGTAGGCG 60

GTAGGGCC 68

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTCCATTTAA ACTACATGTA GTTTACCATG GGGAACGACT TAGCAATTCC ATCCGCCATC 60

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAAGACTCCG CGGGCGAAGT T 21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTATGGATCC TTAACTAGTG ATTCTAGAGG GCCCCGGCCC GTCGTTGGTC CAACCTTCAT 60

CGGT 64

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3754 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

BNSDOCID: <WO___9504151A2_I_>

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTCAGGTAAA TTTGATGTAC ATCAAATGGT ACCCCTTGCT GAATCGTTAA GGTAGGCGGT 60
AGGGCCCAGA TCTTAATCAT CCACAGGAGA CTTTCTGATG AAAAACCTTG ATTGTTGGGT 120
CGACAACGAA GAAGACATCG ATGTTATCCT GAAAAAGTCT ACCATTCTGA ACTTGGACAT 180
CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA 240
TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC 300
TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC 360
CGTTAGCTTC TGGCTGCGCG TTCCGAAAGT TTCTGCTTCC CACCTGGAAC AGTACGGCAC 420
TAACGAGTAC TCCATCATCA GCTCTATGAA GAAACACTCC CTGTCCATCG GCTCTGGTTG 480
GTCTGTTTCC CTGAAGGGTA ACAACCTGAT CTGGACTCTG AAAGACTCCG CGGGCGAAGT 540
TCGTCAGATC ACTTTCCGCG ACCTGCCGGA CAAGTTCAAC GCGTACCTGG CTAACAAATG 600
GGTTTTCATC ACTATCACTA ACGATCGTCT GTCTTCTGCT AACCTGTACA TCAACGGCGT 660
TCTGATGGGC TCCGCTGAAA TCACTGGTCT GGGCGCTATC CGTGAGGACA ACAACATCAC 720
TCTTAAGCTG GACCGTTGCA ACAACAACAA CCAGTACGTA TCCATCGACA AGTTCCGTAT 780
CTTCTGCAAA GCACTGAACC CGAAAGAGAT CGAAAAACTG TATACCAGCT ACCTGTCTAT 840
CACCTTCCTG CGTGACTTCT GGGGTAACCC GCTGCGTTAC GACACCGAAT ATTACCTGAT 900
CCCGGTAGCT TCTAGCTCTA AAGACGTTCA GCTGAAAAAC ATCACTGACT ACATGTACCT 960
GACCAACGCG CCGTCCTACA CTAACGGTAA ACTGAACATC TACTACCGAC GTCTGTACAA 1020
CGGCCTGAAA TTCATCATCA AACGCTACAC TCCGAACAAC GAAATCGATT CTTTCGTTAA 1080
ATCTGGTGAC TTCATCAAAC TGTACGTTTC TTACAACAAC AACGAACACA TCGTTGGTTA 1140
CCCGAAAGAC GGTAACGCTT TCAACAACCT GGACAGAATT CTGCGTGTTG GTTACAACGC 1200
TCCGGGTATC CCGCTGTACA AAAAAATGGA AGCTGTTAAA CTGCGTGACC TGAAAACCTA 1260
CTCTGTTCAG CTGAAACTGT ACGACGACAA AAACGCTTCT CTGGGTCTGG TTGGTACCCA 1320
CAACGGTCAG ATCGGTAACG ACCCGAACCG TGACATCCTG ATCGCTTCTA ACTGGTACTT 1380
CAACCACCTG AAAGACAAAA TCCTGGGTTG CGACTGGTAC TTCGTTCCGA CCGATGAAGG 1440

TTGGACCAAC GACGGGCCGG GGCCCTCTAG AATCACTAGT TAAGGATCCG CTAGCCCGCC 1500

TAATGAGCGG GCTTTTTTTT CTCGGGCAGC GTTGGGTCCT GGCCACGGGT GCGCATGATC 1560

GTGCTCCTGT CGTTGAGGAC CCGGCTAGGC TGGCGGGGTT GCCTTACTGG TTAGCAGAAT 1620

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GCAACAACAT GAATGGTCTT CGGTTTCCGT GTTTCGTAAA GTCTGGAAAC GCGGAAGTCA 1740

GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC 1800

GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG 1860

AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT 1920

GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA 1980

GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT 2040

CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC 2100

GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT 2160

TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC 2220

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CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG 2340

GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC 2400

AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA CCGCTGGTAG 2460

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CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC 3480

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ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG 3600

CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC 3660

CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC ATGACATTAA CCTATAAAAA 3720

TAGGCGTATC ACGAGGCCCT TTCGTCTTCA AGAA 3754

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TAGTCTAGAA TGGCTGGCGA GCATATCAAG 30

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TTAGGATCCT TAGAAGGGAG TTGCAGGCCT 30

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4378 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTCAGGTAAA TTTGATGTAC ATCAAATGGT ACCCCTTGCT GAATCGTTAA GGTAGGCGGT 60

TET C GENE START CODON
AGGGCCCAGA TCTTAATCAT CCACAGGAGA CTTTCTGATG AAAAACCTTG ATTGTTGGGT 120

CGACAACGAA GAAGACATCG ATGTTATCCT GAAAAAGTCT ACCATTCTGA ACTTGGACAT 180

CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA 240

TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC 300

TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC 360

CGTTAGCTTC TGGCTGCGCG TTCCGAAAGT TTCTGCTTCC CACCTGGAAC AGTACGGCAC 420

TAACGAGTAC TCCATCATCA GCTCTATGAA GAAACACTCC CTGTCCATCG GCTCTGGTTG 480

SacII
GTCTGTTTCC CTGAAGGGTA ACAACCTGAT CTGGACTCTG AAAGACTCCG CGGGCGAAGT 540

TCGTCAGATC ACTTTCCGCG ACCTGCCGGA CAAGTTCAAC GCGTACCTGG CTAACAAATG 600

GGTTTTCATC ACTATCACTA ACGATCGTCT GTCTTCTGCT AACCTGTACA TCAACGGCGT 660

TCTGATGGGC TCCGCTGAAA TCACTGGTCT GGGCGCTATC CGTGAGGACA ACAACATCAC 720

TCTTAAGCTG GACCGTTGCA ACAACAACAA CCAGTACGTA TCCATCGACA AGTTCCGTAT 780

CTTCTGCAAA GCACTGAACC CGAAAGAGAT CGAAAAACTG TATACCAGCT ACCTGTCTAT 840

CACCTTCCTG CGTGACTTCT GGGGTAACCC GCTGCGTTAC GACACCGAAT ATTACCTGAT 900

CCCGGTAGCT TCTAGCTCTA AAGACGTTCA GCTGAAAAAC ATCACTGACT ACATGTACCT 960

GACCAACGCG CCGTCCTACA CTAACGGTAA ACTGAACATC TACTACCGAC GTCTGTACAA 1020

CGGCCTGAAA TTCATCATCA AACGCTACAC TCCGAACAAC GAAATCGATT CTTTCGTTAA 1080

ATCTGGTGAC TTCATCAAAC TGTACGTTTC TTACAACAAC AACGAACACA TCGTTGGTTA 1140

CCCGAAAGAC GGTAACGCTT TCAACAACCT GGACAGAATT CTGCGTGTTG GTTACAACGC 1200

TCCGGGTATC CCGCTGTACA AAAAAATGGA AGCTGTTAAA CTGCGTGACC TGAAAACCTA 1260

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CAACGGTCAG ATCGGTAACG ACCCGAACCG TGACATCCTG ATCGCTTCTA ACTGGTACTT 1380

CAACCACCTG AAAGACAAAA TCCTGGGTTG CGACTGGTAC TTCGTTCCGA CCGATGAAGG 1440

HINGE DOMAIN XbaI S.Mansoni P28 GENE START

TTGGACCAAC GACGGGCCGG GGCCCTCTAG AATGGCTGGC GAGCATATCA AGGTTATCTA 1500

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CGGACGATTG CCTGCAGTGA AAGTCACTGA TGATCATGGG CACGTGAAAT GGATGTTAGA 1680

GAGTTTGGCT ATTGCACGGT ATATGGCGAA GAAACATCAT ATGATGGGTG AAACAGACGA 1740

GGAATACTAT AGTGTTGAAA AGTTGATTGG TCATGCTGAA GATGTAGAAC ATGAATATCA 1800

CAAAACTTTG ATGAAGCCAC AAGAAGAGAA AGAGAAGATA ACCAAAGAGA TATTGAACGG 1860

CAAAGTTCCA GTTCTTCTCA ATATGATCTG CGAATCTCTG AAAGGGTCGA CAGGAAAGCT 1920

GGCTGTTGGG GACAAAGTAA CTCTAGCTGA TTTAGTCCTG ATTGCTGTCA TTGATCATGT 1980

GACTGATCTG GATAAAGGAT TTCTAACTGG CAAGTATCCT GAGATCCATA AACATCGAGA 2040

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STOP BamHI

CTTCTAAGGA TCCGCTAGCC CGCCTAATGA GCGGGCTTTT TTTTCTCGGG CAGCGTTGGG 2160

TCCTGGCCAC GGGTGCGCAT GATCGTGCTC CTGTCGTTGA GGACCCGGCT AGGCTGGCGG 2220

GGTTGCCTTA CTGGTTAGCA GAATGAATCA CCGATACGCG AGCGAACGTG AAGCGACTGC 2280

BNSDOCID: <WO__9504151A2_I_>

TGCTGCAAAA CGTCTGCGAC CTGAGCAACA ACATGAATGG TCTTCGGTTT CCGTGTTTCG 2340

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CTCGGTCGTT CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC 2460

CACAGAATCA GGGGATAACG CAGGAAAGAA CATGTGAGCA AAAGGCCAGC AAAAGGCCAG 2520

GAACCGTAAA AAGGCCGCGT TGCTGGCGTT TTTCCATAGG CTCCGCCCCC CTGACGAGCA 2580

TCACAAAAAT CGACGCTCAA GTCAGAGGTG GCGAAACCCG ACAGGACTAT AAAGATACCA 2640

GGCGTTTCCC CCTGGAAGCT CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG 2700

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GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG AACCCCCCGT 2820

TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA 2880

CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG 2940

CGGTGCTACA GAGTTCTTGA AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT 3000

TGGTATCTGC GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA GCTCTTGATC 3060

CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC AGATTACGCG 3120

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GTCTGACAGT TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG 3360

TTCATCCATA GTTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG AGGGCTTACC 3420

ATCTGGCCCC AGTGCTGCAA TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC 3480

AGCAATAAAC CAGCCAGCCG GAAGGGCCGA GCGCAGAAGT GGTCCTGCAA CTTTATCCGC 3540

CTCCATCCAG TCTATTAATT GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG 3600

TTTGCGCAAC GTTGTTGCCA TTGCTGCAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT 3660

GGCTTCATTC AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG 3720

CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC GATCGTTGTC AGAAGTAAGT TGGCCGCAGT 3780

GTTATCACTC ATGGTTATGG CAGCACTGCA TAATTCTCTT ACTGTCATGC CATCCGTAAG 3840

ATGCTTTTCT GTGACTGGTG AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG 3900

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AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA TCTTACCGCT 4020

GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC TGATCTTCAG CATCTTTTAC 4080

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TTATCAGGGT TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA 4260

AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG AAACCATTAT 4320

TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC TTCAAGAA 4378

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAAGACTCCG CGGGCGAAGT T

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

BNSDOCID: <WO__9504151A2_I_>

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TTATCTAGAG TCGTTGGTCC AACCTTCATC

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4366 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TTCAGGTAAA TTTGATGTAC ATCAAATGGT ACCCCTTGCT GAATCGTTAA GGTAGGCGGT 60

TET C GENE START CODON

AGGGCCCAGA TCTTAATCAT CCACAGGAGA CTTTCTGATG AAAAACCTTG ATTGTTGGGT 120

CGACAACGAA GAAGACATCG ATGTTATCCT GAAAAAGTCT ACCATTCTGA ACTTGGACAT 180

CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA 240

TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC 300

TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC 360

CGTTAGCTTC TGGCTGCGCG TTCCGAAAGT TTCTGCTTCC CACCTGGAAC AGTACGGCAC 420

TAACGAGTAC TCCATCATCA GCTCTATGAA GAAACACTCC CTGTCCATCG GCTCTGGTTG 480

SacII

GTCTGTTTCC CTGAAGGGTA ACAACCTGAT CTGGACTCTG AAAGACTCCG CGGGCGAAGT 540

TCGTCAGATC ACTTTCCGCG ACCTGCCGGA CAAGTTCAAC GCGTACCTGG CTAACAAATG 600

GGTTTTCATC ACTATCACTA ACGATCGTCT GTCTTCTGCT AACCTGTACA TCAACGGCGT 660

TCTGATGGGC TCCGCTGAAA TCACTGGTCT GGGCGCTATC CGTGAGGACA ACAACATCAC 720

TCTTAAGCTG GACCGTTGCA ACAACAACAA CCAGTACGTA TCCATCGACA AGTTCCGTAT 780

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XbaI S.Mansoni P28 GENE START

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STOP BamHI

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TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC 4260

GCGCACATTT CCCCGAAAAG TGCCACCTGA CGTCTAAGAA ACCATTATTA TCATGACATT 4220

AACCTATAAA AATAGGCGTA TCACGAGGCC CTTTCGTCTT CAAGAA 4366

CLAIMS

1. A DNA construct comprising a DNA sequence encoding a fusion protein of the formula TetC-$(Z)_a$-Het, wherein TetC is the C fragment of tetanus toxin, or a protein comprising the epitopes thereof; Het is a heterologous protein, Z is an amino acid, and a is zero or a positive integer, provided that $(Z)_a$ does not include the sequence Gly-Pro.

2. A DNA construct according to Claim 1 wherein $(Z)_a$ is a chain of 0 to 15 amino acids.

3. A DNA construct according to Claim 2 wherein $(Z)_a$ is a chain of less than 4 amino acids.

4. A DNA construct according to Claim 3 wherein $(Z)_a$ is a chain of two or three amino acids, the DNA sequence for which defines a restriction endonuclease cleavage site.

5. A DNA construct according to Claim 2 wherein a is zero.

6. A DNA construct according to Claim 2 in which $(Z)_a$ is free from glycine and/or proline.

7. A DNA c nstruct according t any one of the preceding

Claims wherein the heterologous protein Het is an antigenic sequence derived from a virus, bacterium, fungus, yeast or parasite.

8. A DNA construct according to Claim 7 wherein the heterologous protein Het is the Schistosoma mansoni P28 glutathione *S*-transferase antigen.

9. A replicable expression vector, for example suitable for use in bacteria, containing a DNA construct as defined in any one of Claims 1 to 8.

10. A host, for example, a bacterium, having integrated into the chromosomal DNA thereof a DNA construct as defined in any one of Claims 1 to 8.

11. A fusion protein as defined in any one of Claims 1 to 8.

12. A process for the preparation of a bacterium (preferably an attenuated bacterium), which process comprises transforming a bacterium with a DNA construct as defined in any one of Claims 1 to 8.

13. A vaccine composition comprising a fusion protein, or an attenuated bacterium expressing said fusion protein, the fusion protein being as defined in any one of Claims 1 to 8; and a pharmaceutically

acceptable carri r.

14. A method of immunising a patient, e.g. a human patient, which comprises administering to the patient an effective immunising amount of a vaccine composition as defined in Claim 13.

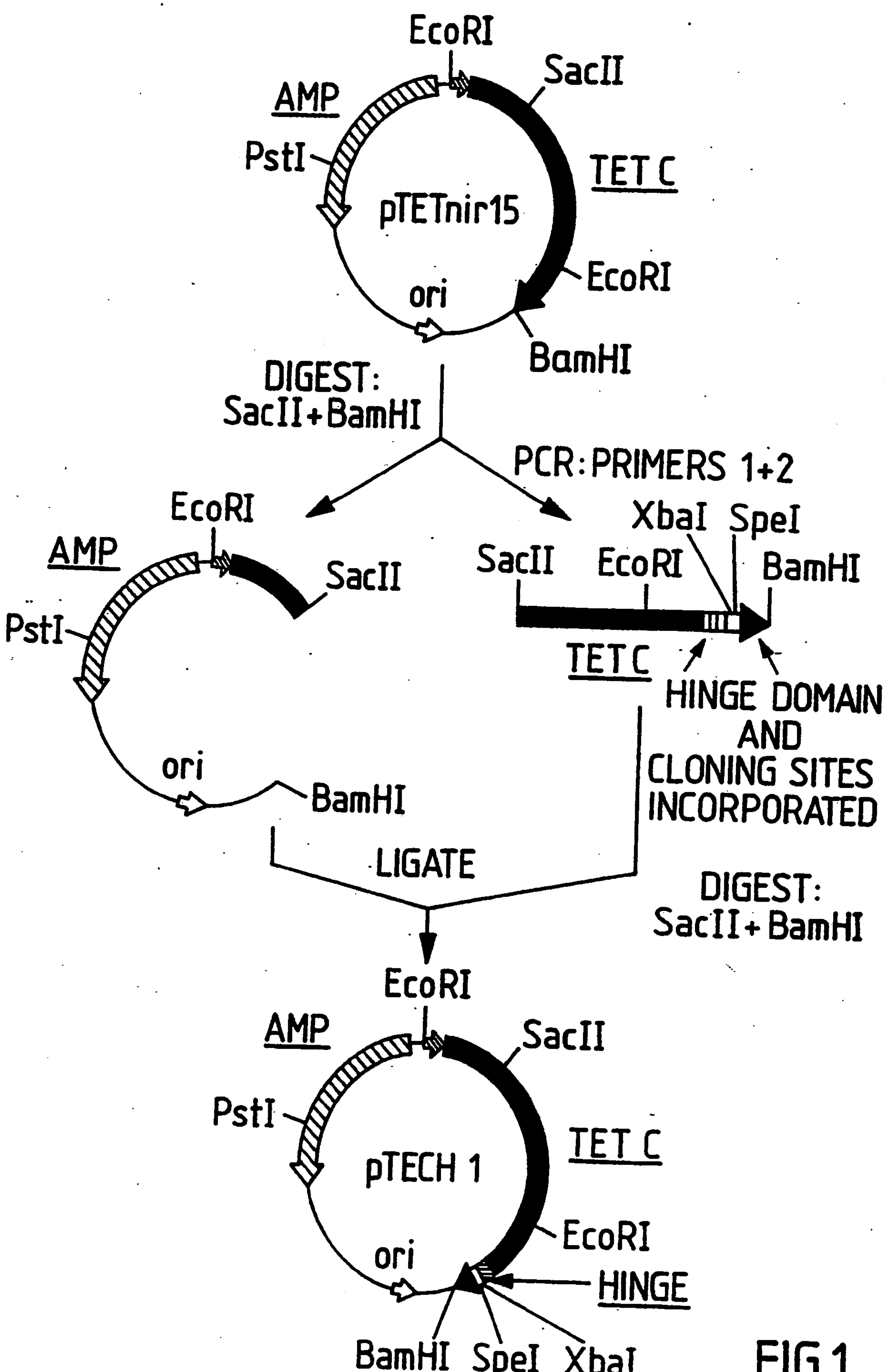

FIG.1

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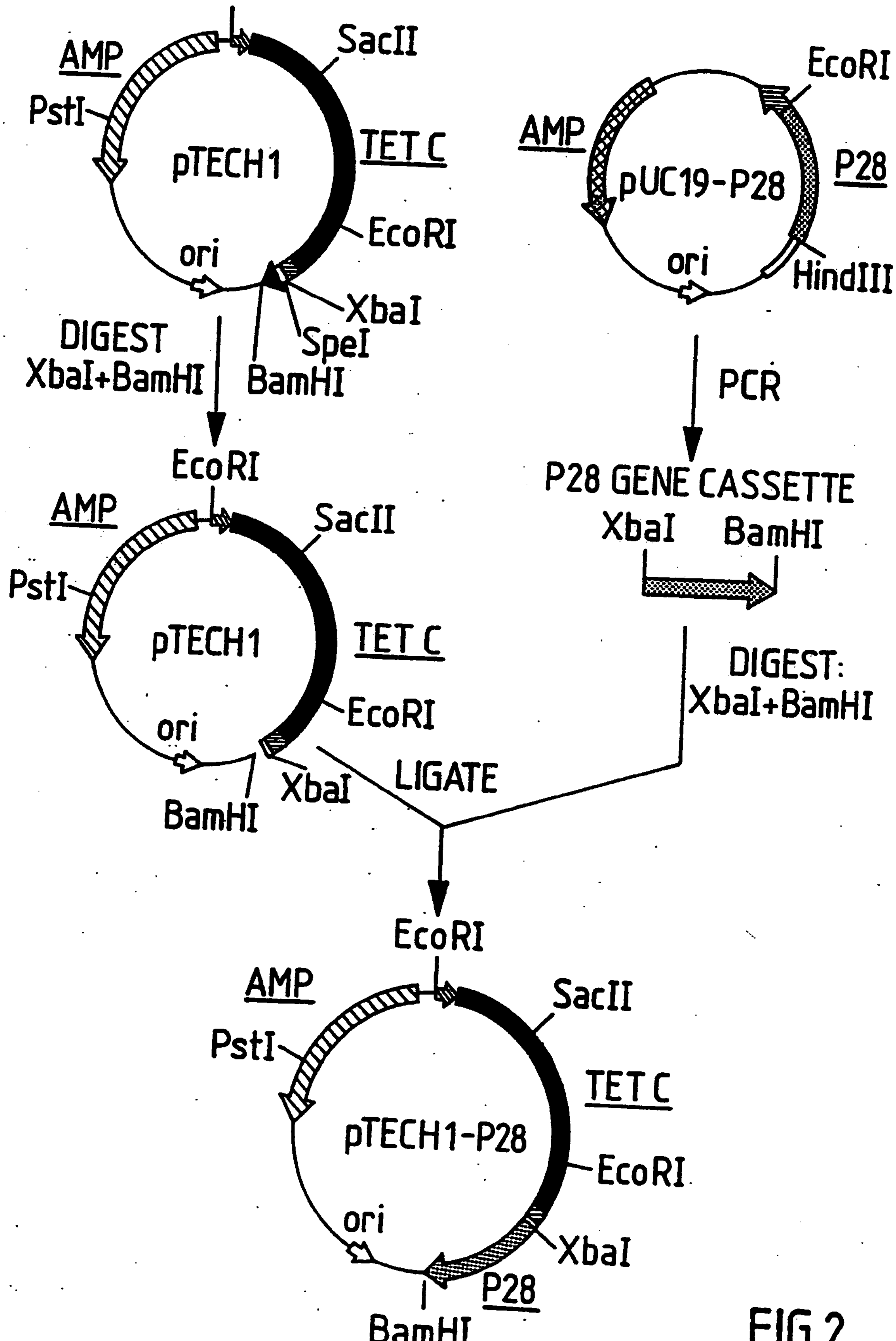
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PstI
pTECH1
SacII
TET C
EcoRI
ori
XbaI
SpeI
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XbaI+BamHI
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EcoRI
P28
ori
HindIII
PCR
P28 GENE CASSETTE
XbaI
BamHI
EcoRI
AMP
PstI
pTECH1
SacII
TET C
EcoRI
ori
BamHI
XbaI
LIGATE
DIGEST:
XbaI+BamHI
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BamHI

FIG.2

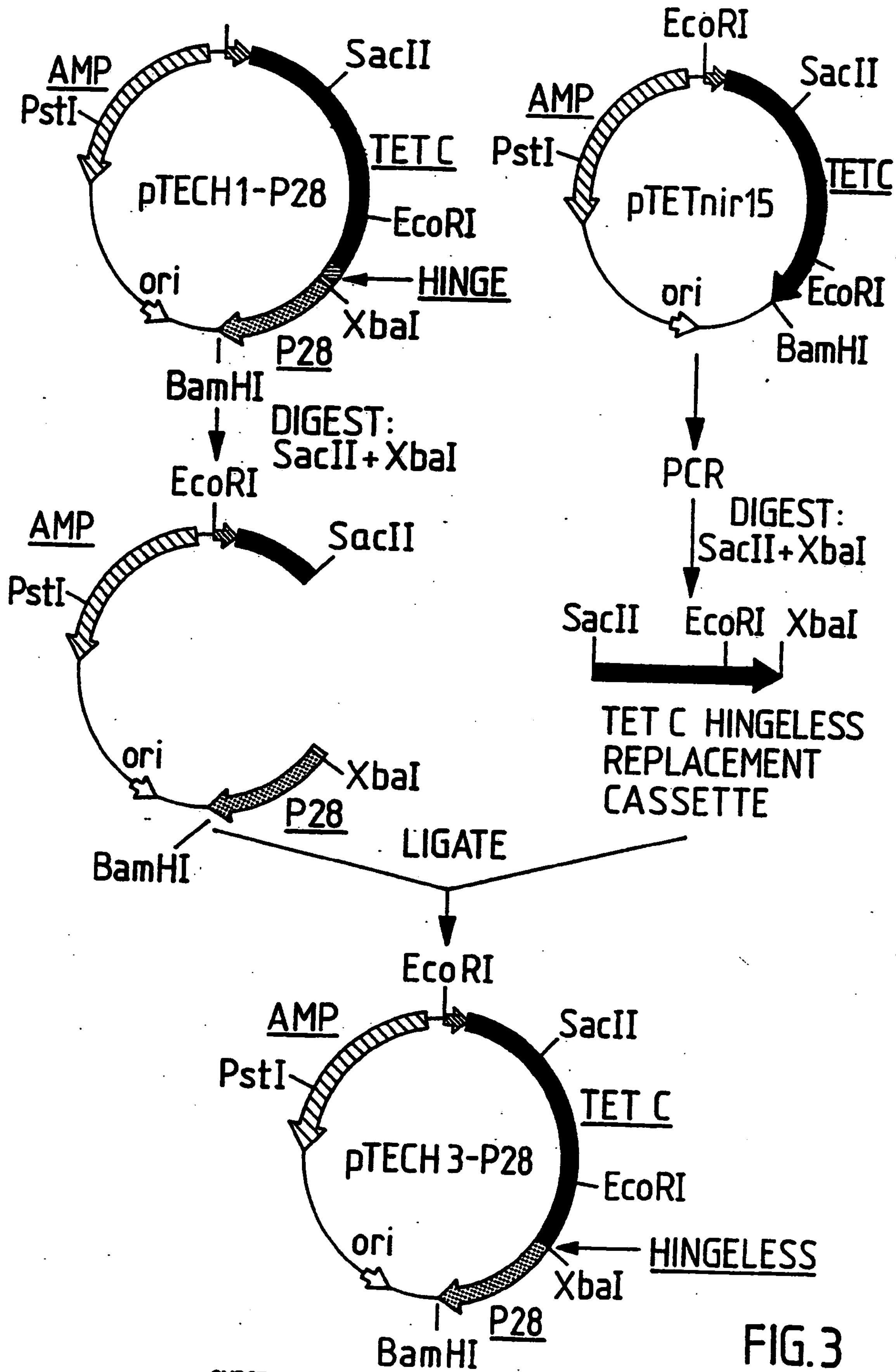

FIG.3

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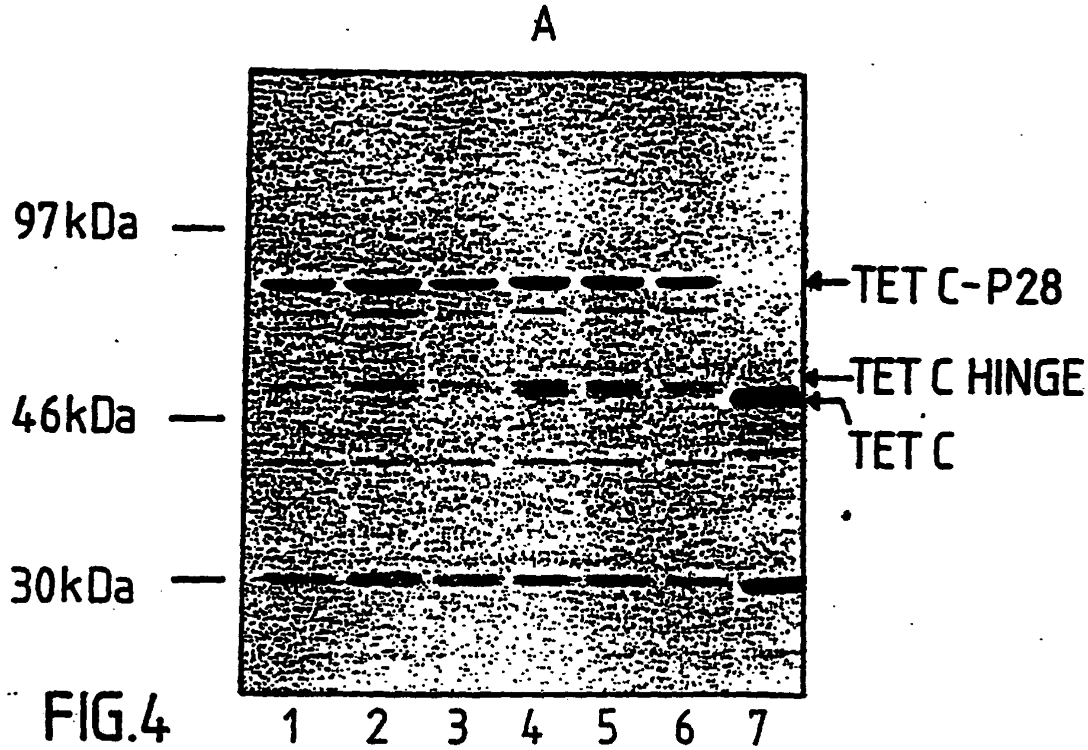

FIG.4

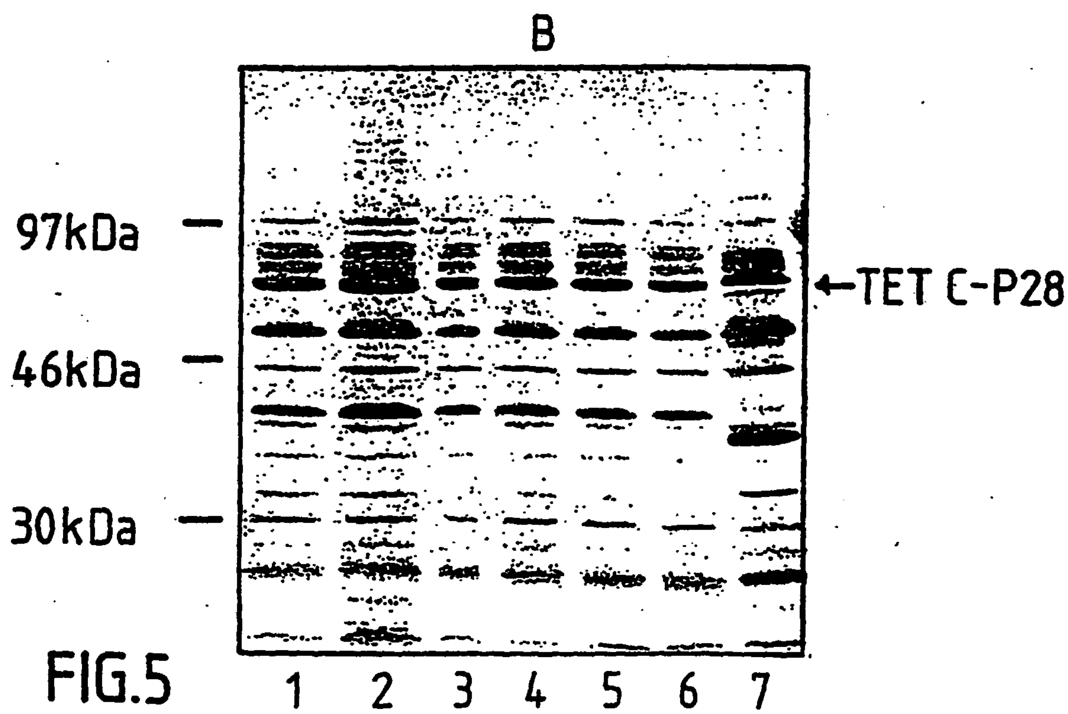

FIG.5

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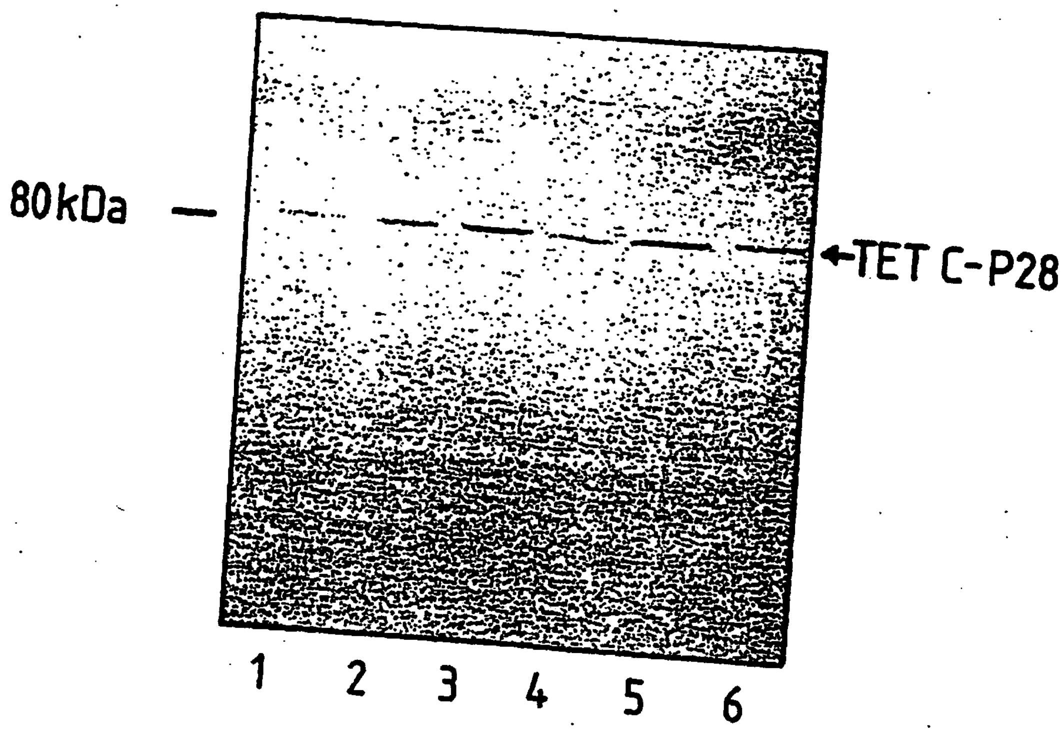

FIG.6

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/62, 15/31, 15/54, 1/21, A61K 38/45 // (C12N 1/21, C12R 1:42)

A3

(11) International Publication Number: WO 95/04151

(43) International Publication Date: 9 February 1995 (09.02.95)

(21) International Application Number: PCT/GB94/01647

(22) International Filing Date: 29 July 1994 (29.07.94)

(30) Priority Data:
PCT/GB93/01617 30 July 1993 (30.07.93) WO
(34) Countries for which the regional or international application was filed: GB et al.
9401787.8 31 January 1994 (31.01.94) GB

(71) Applicant *(for all designated States except US)*: MEDEVA HOLDINGS B.V. [NL/NL]; Churchill-Laan 223, NL-1078 ED Amsterdam (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KHAN, Mohammed, Anjam [GB/GB]; Cambridge University Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB). HORMAECHE, Carlos, Estenio [GB/GB]; Cambridge University Dept. of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB). CHATFIELD, Steven, Neville [GB/GB]; Medeva Vaccine Research Unit, Dept. of Biochemistry, Imperial College of Science and Technology, London SW7 2AY (GB). DOUGAN, Gordon [GB/GB]; Medeva Vaccine Research Unit, Dept. of Biochemistry, Imperial College of Science and Technology, London SW7 2AY (GB).

(74) Agents: HUTCHINS, Michael, Richard et al.; Fry Heath & Spence, St. Georges House, 6 Yattendon Road, Horley, Surrey RH6 7BS (GB).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

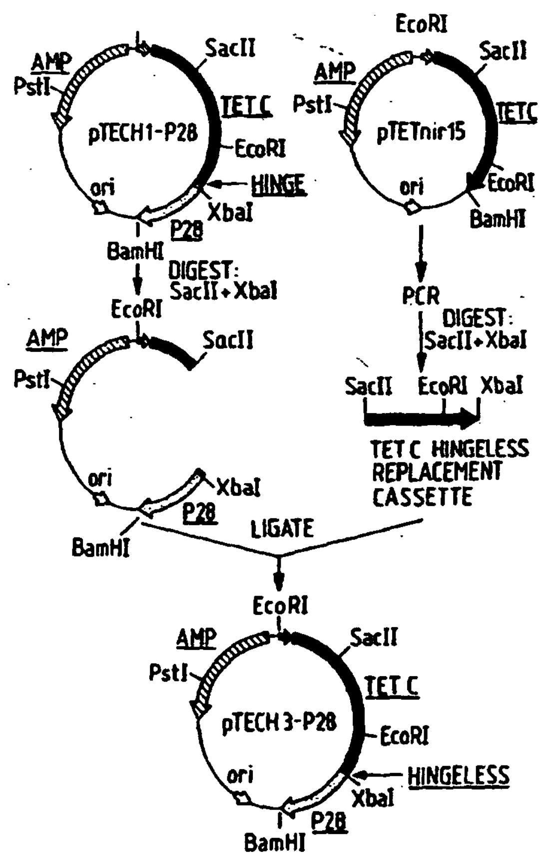
Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report: 16 March 1995 (16.03.95)

(54) Title: VACCINE COMPOSITIONS CONTAINING RECOMBINANT TETC-FUSION PROTEINS

(57) Abstract

The invention provides a DNA construct comprising a DNA sequence encoding a fusion protein of the formula: TetC-$(Z)_a$-Het, wherein: TetC is the C fragment of tetanus toxin, or a protein comprising the epitopes thereof; Het is a heterologous protein, Z is an amino acid, and a is zero or a positive integer, provided that $(Z)_a$ does not include the sequence Gly-Pro. The invention also provides replicable expression vectors containing the constructs, bacteria transformed with the constructs, the fusion proteins *per se* and vaccine compositions formed from the fusion proteins or attenuated bacteria expressing the fusion proteins.



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A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/62 C12N15/31 C12N15/54 C12N1/21 A61K38/45
//(C12N1/21,C12R1:42)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,89 06974 (PRAXIS BIOLOGICS, INC.) 10 August 1989 see page 25, line 12 - page 26, line 9 see page 26, line 25 - page 27, line 14 see page 76, line 1 - page 81, line 4; claims 1-107; tables 12-15	1-14
Y	EP,A,0 432 965 (SMITHKLINE BEECHAM) 19 June 1991 see page 3, line 49 - line 58 see page 5, line 2 - line 4 see page 16, line 24 - line 29	1-14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 January 1995	[illegible] 02 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIO/TECHNOLOGY, vol.10, no.8, August 1992, NATURE AMERICA, INC., NEW YORK, US; pages 888 - 892 S.N. CHATFIELD ET AL. 'Use f the nirB promoter to direct the stable expression of heterologous antigens in Salmonella oral vaccine strains: Development of a single-dose oral tetanus vaccine' see page 888, right column, line 22 - page 889, left column, line 28 see page 891, left column, line 39 - line 46	1-14
Y	WO,A,92 15689 (THE WELLCOME FOUNDATION LIMITED) 17 September 1992 cited in the application see page 8, line 2 - page 9, line 26; claims 1-12 see page 11, line 20 - line 35	1-14
Y	J. IMMUNOLOGY, vol.141, no.5, 1 September 1988, AM. SOC. IMMUNOLOGISTS,US; pages 1687 - 1694 C. AURIAULT ET AL. 'Analysis of T and B cell epitopes of the Schistosoma mansoni P28 antigen in the rat model by using synthetic peptides' see page 1688, left column, line 18 - line 29	1-14
A	NUCLEIC ACIDS REASEARCH, vol.19, no.11, 11 June 1991, IRL,OXFORD UNIVERSITY PRESS, UK; pages 2889 - 2892 M.D. OXER ET AL. 'High level heterologous expression in E. coli using the anaerobically-activated nirB promoter' see page 2890, left column, paragraph 4 - page 2892, right column, line 19	1-14
A	WO,A,91 09621 (INSTITUT PASTEUR) 11 July 1991 the whole document	1-14
A	WO,A,93 08290 (UNIVERSITY OF SASKATCHEVAN) 29 April 1993 see page 3, line 32 - page 5, line 2 see page 16, line 29 - page 17, line 7	1-14

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FEMS SYMPOSIUM, NO. 73. BACTERIAL PROTEIN TOXINS; GUSTAV FISCHER VERLAG: STUTTGART, GERMANY; 0 (0). 1994 FREER, J. ET AL (ED.) 6th European Workshop, Stirling, Scotland, UK, June 27- July 2, 1993; the whole document ---	1-14
P,X, L	WO,A,94 03615 (MEDEVA HOLDINGS B.V.) 17 February 1994 cited in the application see claims 1-24 -----	1-14

2

INT[redacted]ATIONAL SEARCH REPORT

International application No.
PCT/GB94/01647

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Alth ugh claim 14 is directed to a method f treatment of the human b dy the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

[illegible] patent family members

PCT/[illegible]4/01647

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8906974	10-08-89	AT-T-	109008	15-08-94
		AU-B-	634153	18-02-93
		AU-A-	3065489	25-08-89
		DE-D-	68917126	01-09-94
		EP-A-	0399001	28-11-90
		JP-T-	3502691	20-06-91
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		WO-A-	9215688	17-09-92
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WO-A-9403615	17-02-94	AU-B-	4719393	03-03-94

NSDOCID: <WO___9504151A3_I_>